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An Experimental Test of the Relationship Between Temporal Variability of Feeding Opportunities and Baseline Levels of Corticosterone in a Shorebird

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ABSTRACT In this study, we tested the hypothesis that baseline corticosterone levels increase with a change from constant to variable feeding schedules. Captive red knots, *Calidris canutus*, were presented with food that was either available during the same time each day (constant) or starting at variable times during the day. Food intake rates, frequency of aggressive interactions, and baseline levels of corticosterone were measured. In the majority of cases, red knots showed higher plasma corticosterone concentrations during feeding schedules that were irregular than when food was available at consistent times. These findings are supported by a previous study that showed that red knots take a long time to adjust to the newly offered, predictable conditions of their aviary environment. The frequency of conflicts in the different groups and (size-corrected) body mass were not correlated with average corticosterone level. The results are examined in the light of literature showing that increases in corticosterone in response to acute, unpredictable events mediate behavioral responses such as increased explorative behavior and memory. For red knots that have to find their food on the temperate-zone mudflats in Western Europe, an increased circulating corticosterone level may be adaptive during periods when the patchily distribution of buried bivalves and the burying behavior of such prey presents them with a variable and unpredictable food supply. *J. Exp. Zool. 293:81–88, 2002.*

Unpredictable environmental conditions induce elevated corticosterone concentrations, which in turn may mediate behavioral and physiological responses to overcome the negative impact of the environmental stressor (e.g., Wingfield, '94; Wingfield and Ramenofsky, '99). Elevated, baseline levels of corticosterone have been related to increased foraging, exploratory behavior, and enhanced memory during environmental disturbances (Stuebe and Ketterson, '82; Astheimer et al., '92; Breuner, '98; Saldanha et al., 2000). Environmental unpredictability is often described as a single, often acute, event such as a sudden attack by a predator or the occurrence of a snow-storm that, for hours to days, disrupts 'normal' ongoing activities by temporally diminishing food resources and by increasing energetic demands (Wingfield and Ramenofsky, '99). In this study on a shorebird species (red knot, *Calidris canutus*), we tested the hypothesis that baseline levels of corticosterone rise during periods when the start of time intervals of food presentation varied unpredictably rather than being fixed.

An earlier study on long-term, baseline concentrations of corticosterone showed that red knots maintain elevated baseline concentrations of corticosterone during their first year of captivity (Piersma and Ramenofsky, '98). The unpredictability of their captive environment is much lower than in the field. Although not necessarily life threatening or stressful, persistent environmental unpredictability would require behavioral responses known to be facilitated by increased corticosterone levels during acute, unpredictable events. It was argued that birds might require a full year (in which they experience a complete set of life history stages;

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Jacobs and Wingfield, 2000) to fully adjust to confinement.

Temporal variability of the availability of food may elicit similar adjustments in behavior and physiology— adjustments that may be mediated by elevated baseline levels of corticosterone. When the temporal and spatial distributions of prey are known and/or change predictably, birds can anticipate by focusing foraging efforts on times and places that are most likely to be successful. Hen harriers, *Circus cyanus*, and kestrels, *Falco tinnunculus*, focus their hunting activity on times of the day when their prey show the most surface activity and are most easily discovered and captured (Daan and Aschoff, '82). Kestrels will revisit previously successful hunting sites (Rijnsdorp et al., '81).

Prey availability, however, will more often vary unpredictably in space and time. Such variation may compromise anticipatory activities on the part of the predator. Animals subjected to variable feeding conditions have to adjust their behavior. Such coping may include increased exploration of the home range to gain knowledge about (changes in) temporal and spatial food distribution (e.g., Giraldeau, '97). For example, during the nonbreeding season, red knots use larger foraging areas on the mudflats in Western Europe compared to conspecifics wintering in the tropical mudflats of Western Africa where the availability of prey items is likely to be more constant (Piersma et al., '93). This could reflect an increase in the sustained exploratory behavior and may help the northern wintering populations to keep track of the locations of good feeding patches. Variable food conditions can also be met with increased food intake and storage of fat as a buffer against periods of shortage (Bednekoff and Krebs, '95; Cuthill et al., 2000). Corticosterone has been shown to facilitate such responses (Astheimer et al., '92; Wingfield and Silverin, '86).

Long-term high corticosterone concentrations in response to stressful events can have many detrimental effects (e.g., breakdown of skeletal muscle, Cherel et al., '88, Dallman et al., '93; impaired memory, McEwen and Sapolsky, '95). On the other hand, short-term and intermediate corticosterone levels are expected to mediate different behavioral and physiological adjustments that may be advantageous (reviewed by Wingfield and Ramenofsky, '99). We hypothesized that corticosterone facilitates behaviors that reduce the impact of uncertainty (stress). We therefore expected baseline concentrations of corticosterone, rather than stress-induced corticosterone levels, to be higher during variable conditions. To test this hypothesis, we fed captive red knots either at consistent times from day to day or at irregular times. The incidence of relevant behaviors such as aggression and rates of food intake was scored, and baseline concentrations of corticosterone were measured weekly. The invariant length of the daily feeding period ensured that daily food intake per se was similar across experimental treatments.

MATERIALS AND METHODS

Birds and aviaries

Red knots belonging to the subspecies *C. c. islandica* were captured with mist-nets on November 6, 1994 and October 27, 1995 in the western Wadden Sea, The Netherlands. All were older than two years. They were sexed by a molecular assay (Baker et al., '99); only two of the 13 red knots were female and the two females were in different groups. The red knots had been in captivity for at least two years when the experiment started and were thus fully accustomed (cf. Piersma and Ramenofsky, '98). Only three individuals had been used before in a noninvasive experiment in April 1996. These animals occurred in different experimental groups in the study described here. Birds were individually color-banded to be identifiable during visual observations.

The aviaries measured 2 m × 4 m with a height of approximately 2 m and were situated next to each other but were visually separated. The upper half of one side of the aviaries consisted of wire netting through which the birds were exposed to the outdoors. The roof was semi-transparent and light conditions followed the local regime. A quarter of the aviary floors were covered by an artificial sandflat with continuously flowing salt water. The aviaries also had a tray of fresh water for drinking and bathing.

At weekly intervals, birds were taken out of the aviaries for a few hours while the aviaries were cleaned and disinfected. During these periods, their body mass was measured to the nearest gram on an electronic balance. Cage cleaning-related disturbances took place after blood sampling, and interference with corticosterone levels was thus minimal.

Feeding schedules

Experiments took place during February to April 1998 and February to April 1999. The timing of the presentation of food was manipulated by having food available either at a constant or at a variable time of the day. In both the constant and the

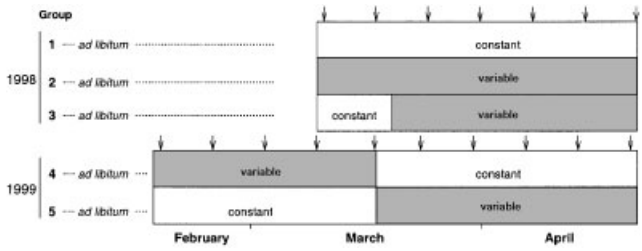

Fig. 1. Timing of constant (unshaded blocks) and variable (shaded blocks) feeding schedules to the different experimental groups of red knots in 1998 and 1999. Arrows (↓) mark the days when body mass was determined and blood samples were taken.

variable feeding schedules, food was available for a total of six hours every day throughout the experiments. This period was sufficient for birds to increase body mass. The birds were fed protein-rich trout food pellets.

In two years, five groups of four to six birds were subjected to either of two contrasting feeding schedules (Fig. 1). In 1998, the birds in the constant regime always had food available between 700 hr and 1300 hr. In the variable regime, the six-hour feeding period started at irregular times between 700 hr and 2200 hr, using sequential values from a table of random number. One experimental group ('group # 1') was fed according to the constant feeding regime throughout the experimental period. A second group ('group # 2') obtained food at variable times during the day throughout the experiment. A third group ('group # 3') was subjected first to the constant feeding regime but was switched to the variable feeding regime on March 19.

In 1999, two new experimental groups were assembled from the individuals studied in 1998. They were assigned such that initial body mass distributions were similar between groups. In 1999, there were two groups of six birds; group # 4 was first fed on a variable time schedule and then, at a set time, and group # 5 had the contrasting program. The two groups in 1999 were given contrasting schedules to test for any time effect. The switch was on March 17. During the control treatment, birds had access to food between 1300 hr and 1900 hr, and the variable feeding period of six hours was started between 700 hr and 1900 hr. Experiments ended in both years on April 21 before birds started pre-migratory fuelling that, in captive red knots, typically starts at the end of April/beginning of May. Possible effects of increased fuel stores on baseline levels of corticosterone (cf. Piersma et al., 2000) were therefore excluded. Before and after the experiments, food regimes were ad libitum.

In 1998, food was offered on ca. 30 cm-high platforms accessed by a footbridge. This narrow approach of the food tray may have influenced the number of conflicts between individuals. In 1999, we tried to avoid conflicts by spacing three feeding trays evenly over the aviary. Placement and withdrawal of food trays necessitated that a person entered the aviary. We tried to prevent disturbance by behaving as inconspicuously as possible.

We started with a pre-experimental period of three weeks during which the birds were trained to feed on the platforms and could get used to the experimental feeding schedules. As, even when working with teams of three to five persons, only a limited number of birds can be bled within the time span of four minutes after cage entry, we kept the number of experimental birds rather low. During the pre-experimental period we observed the red knots carefully and weighed them daily to ensure that all individuals received enough food. After a drop in body mass the first day after the reduction in the available feeding time, all birds slowly regained their mass over a period of less than a week. Although many conflicts occurred between individuals during the pre-experimental period, these did not prevent individuals from feeding. Individuals might threaten each other, but there was no physical contact.

Throughout the experiments, all individual birds maintained or even increased body mass. For example, even the lowest weighing bird in 1998 (103 g on average) maintained a stable body mass, ranging from 99 g to 107 g during the experiment. Experiments were carried out under auspices of the Animal Experiment Committee (DEC) of the Dutch Royal Academy of Sciences (KNAW) and conform to NIH guidelines.

Behavioral observations

In 1998, behavioral observations were conducted almost daily during the first four hours after food was placed in the aviary. Days with four-hour observation periods were spread evenly between different aviaries. During the observations, the number of ingested pellets and duration of feeding bouts were determined for each individual bird. In addition, conflicts between individuals were recorded. As a measure of aggressive interactions in the different aviaries, we used the frequency of conflicts initiated by individual birds. A conflict was defined as any aggressive behavior exhibited by an individual that displaced another. These were mainly conflicts over food that occurred close to the feeding

tray. A constant dim light in the evening and at night enabled nocturnal observations.

In 1999, no standardized behavioral observations were performed, but we observed the birds daily at random time points, for up to a total of half an hour per aviary each day, and thus checked whether conflicts occurred.

Blood sampling

Before the start of the weekly cleaning of the aviaries, we captured the red knots from the aviaries to collect blood samples of 70–200 µl from their wing vein into one to four hematocrit capillary tubes. Days of blood sampling are marked with arrows in Fig. 1. We always started the bleeding procedure between 900 hr and 1100 hr on the different days of blood sampling to avoid any effects of possible diurnal cycles in corticosterone levels (cf. Joseph and Meier, '73; Marra et al., '95; Breuner et al., '99). The order of cage-entry differed between sampling days to minimize influences of possibly evoking stress responses in neighboring cages. In 1998, birds in the constant treatment were always bled when food was available. By chance, all randomly fed birds were without food at the time blood sampling occurred. To rectify this problem, in 1999, bleedings were timed such that experimental birds had always been without food for at least eight hours.

Immediately after blood sampling, plasma was separated from red blood cells by centrifugation for ten minutes at 6,900 *g*. Plasma was stored frozen at -80°C until transport on dry ice to Seattle, WA. Because we were not interested in the acute levels of corticosterone induced by stress of capture and handling (cf. Wingfield, '94), we only used samples taken within four minutes after cage entry (cf. Piersma et al., 2000). Samples collected within this time limit are considered 'baseline' and reflect the response to living conditions.

Radio-immuno assay (RIA)

Concentration of corticosterone was determined by specific RIA according to the procedures described by Wingfield and Farner ('75) but with the modification of Ball and Wingfield ('87). The plasma samples were thawed, and 20 µl of each sample of plasma were pipetted into glass centrifuge tubes to which 180 µl of distilled water was added. A total of 2,000 counts per minute (cpm) (^{3}H)-corticosterone (NEN: NET-399) was added to each sample and allowed to equilibrate overnight at 4°C. This served to assess the percentage of recovery of steroid throughout extraction procedures. The lipid fraction in each sample was extracted with 4 ml of distilled dichloromethane and dried under nitrogen gas at 40°C. The dried extracts were resuspended in 550 µl phosphate-buffered saline with 0.1% gelatin. Subsamples of 200 µl were placed in duplicate assay tubes and committed to the RIA. The remaining 100 µl were pipetted into a glass vial to which 4.5 ml scintillation fluid was added. Counts per minute from each vial were corrected for dilution and provided an estimate for percent recovery of steroid for each sample. Values of each sample were determined from a standard curve that ranged from 7.8 ng/ml to 1,000 ng/ml, and each sample was adjusted for percentage of recovery. The percentages of recovery ranged between 69.8% and 100% for all assays. Values were read in units of ng/ml after correction for dilution was made. A total of five assays were run. Inter- and intra- assay variation was less than 13% and 5%, respectively.

Statistical analysis and comparisons

Units of analysis were individual averages based on weekly data points of the different parameters of experimental treatments. Two-sample heteroscedastic *t*-tests, assuming unequal variances, were used to compare the average individual parameters between the experimental groups in 1998. We used paired *t*-tests to compare averages between treatments within a group (group # 3 in 1998, and group # 4 and group # 5 in 1999).

As only averages of individuals are compared, differences in temporal changes of measured variables between individuals in different aviaries may remain undetected. To test for the effect of food variability on circulating corticosterone, one-tailed tests were used because we predicted that temporally variable feeding regimes would lead to higher corticosterone levels. In all other cases, two-tailed tests were used. Due to the differences in experimental details, no comparisons were made between years.

A multiple linear regression model revealed that, of several body parameters, 'total head' (bill and head together) best predicted differences in body mass with body size ($r^2 = 0.61$). We used this parameter to correct body mass for differences in body size according to the following formula: (body mass*average total head^3)/total head^3. We will refer to this as 'size corrected body mass' (body mass$_{sc}$).

RESULTS

Average body mass$_{sc}$ for birds in group # 2 in 1998 did not differ from group # 1 ($t = 0.785$, $df = 7$, $P = 0.458$) and also did not differ during the

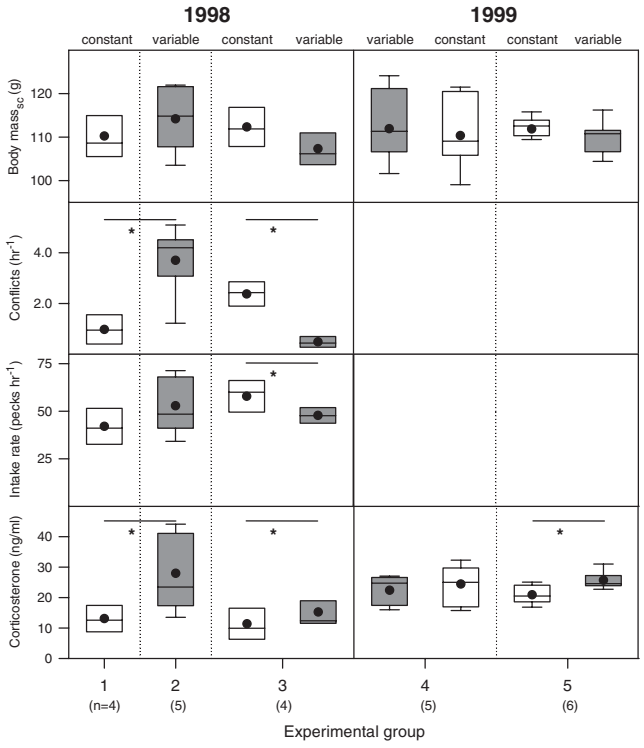

Fig. 2. Summary of treatment differences in size corrected body mass, conflicts, food intake, and circulating corticosterone concentration during constant (unshaded boxes) and variable (shaded boxes) feeding schedules in five experimental groups. The boxes enclose the 50% of the values and the vertical lines show the range of individual averages. Note that there are no vertical lines indicating the range when sample size equals or is less than four, but that the boxes enclose the full range in those cases. The dividing lines within the boxes indicate the median, and the black dots indicate the averages. The number of individual birds studied in each group is given in parentheses on the x-axis. A horizontal line with asterisk marks statistically significant differences.

variable feeding period compared to the constant feeding period in group # 3 (Fig. 2; $t = 2.324$, $df = 4$, $P = 0.081$). Such differences were also absent in 1999 (group # 4: $t = 2.611$, $df = 4$, $P = 0.059$; group # 5: $t = 1.351$, $df = 5$, $P = 0.235$).

In 1998, food intake in group # 2 was not significantly different from group # 1 (Fig. 2; $t = -1.121$, $df = 7$, $P = 0.299$). Average intake in group # 3 was lower during the period when food was offered at variable times ($t = 3.537$, $df = 4$, $P = 0.038$).

The incidence of aggressive interactions differed greatly between treatments and years. The number of aggressive encounters initiated by the red knots was the highest in group # 2 and differed significantly from group # 1 (Fig. 2; $t = -3.509$, $df = 6$, $P = 0.013$). This may not have been related to variability in onset of feeding, as a reversed trend between treatments was found in group # 3 ($t = 4.257$, $df = 4$, $P = 0.024$). Because we made access to food so much easier in 1999, in none of the aviaries did conflicts occurr as frequently in 1999 as in 1998. The last conflict, observed during the daily, nonstandardized observations in 1999, occurred early in the study on February 24 in group # 5 and on March 4 in group # 4.

In three of the four comparisons between treatments, baseline levels of corticosterone were elevated during periods of temporally variable feeding conditions (Fig. 2). Average corticosterone concentrations were higher in the variably fed group # 2 compared to the temporally constantly fed group # 1 ($t = -2.211$, $df = 6$, $P = 0.035$). Also, in experimental group # 3, average baseline corticosterone differed significantly between periods of different feeding schedules ($t = -3.315$, $df = 3$, $P = 0.023$). In 1999, the same applies for corticosterone concentrations of individuals in experimental group # 5 ($t = -3.506$, $df = 5$, $P = 0.009$) but not in group # 4 ($t = -1.300$, $df = 4$, $P = 0.132$). Baseline concentrations of birds in group # 1 that were provided with a temporally constant food source did not differ from baseline concentrations of animals in group # 3 when they received the same treatment ($t = 0.364$, $df = 6$, $P = 0.364$).

DISCUSSION

In three of the four comparisons, plasma corticosterone was significantly higher during experimental variability in food conditions, i.e., consistent with our prediction. The exception occurred in the only group that was given a change from an unpredictable to predictable feeding conditions, after having been exposed to at least two years of ad libitum food supply. Given a previous study that showed red knots to take a full year to reduce corticosterone levels to a constant aviary environment (Piersma and Ramenofsky,'98), we argue that it takes red knots longer to 'recognize' or physiologically adjust to a situation that is temporally constant when they are accustomed to a temporally variable feeding situation than vice versa. In addition, it is possible that once birds experience a constant regime, they 'remember' for quite some time that the possibility remains that conditions may revert, and for precautionary reasons they maintain elevated corticosterone concentrations.

Frequencies of conflicts were not correlated with absence or presence of temporal variability of food in 1998. In 1999, conflicts played no significant role either, but in group # 5, corticosterone concentra-

tion still differed between treatments. In contrast to previous findings during the breeding period (see Sorenson et al. ['97] for the role of glucocorticoids in aggressive conflicts during mate choice), conflicts appear to be independent of corticosterone levels during the nonbreeding period.

Jenni et al. (2000) found elevated corticosterone concentrations in migratory passerines after a long flight, but only in individuals with severely emaciated breast muscles. Such increased corticosterone concentrations in starving individuals may invoke a sudden change in behavior (Cherel et al., '88). In contrast, the red knots showed normal body mass values during the experiments. Furthermore, the lack of a relationship between body $mass_{sc}$ and corticosterone, in both years, renders it unlikely that variation in fuel stores (rather than food variability) explains the difference in corticosterone levels.

In 1998, birds were without food for at least five hours when blood was sampled during the variable feeding regimes. This may have contributed to higher baseline concentrations of corticosterone than in 1999 (cf. Astheimer et al., '92). However, free-living red knots on tidal mudflats are deprived of food for several hours twice a day when the high tide forces birds to refrain from feeding (Zwarts et al., '90). Therefore, we do not expect periods without food of such lengths to have caused additional stress, especially as all individuals were in neutral energy balance. By manipulating the temporal availability of food, one varies the time that birds have to wait for their daily food, and thus possibly a 'motivation to eat.' Of course, the same happens in nature. More frequent instances of 'hunger' may alert them to the fact that food is not as predictably available as before.

Several studies have indicated that increases in circulating corticosterone induce changes in behavior. For example, administration of corticosterone increased apparent escape behavior (perch hopping) in white-crowned sparrows, *Zonotrichia leucophrys gambelii*, and song sparrows, *Melospiza melodia*, in the absence of food (Astheimer et al., '92). Once food was returned, birds increased duration and intensity of feeding. These alterations in behavior in relation to food availability have led to suggestions that corticosterone induces behaviors that favor a positive energy balance (reviewed by Wingfield and Ramenofsky, '99). Our findings did not confirm an increased food intake during variable feeding conditions, possibly because the daily time periods that food was available were equal in both treatments. The lower intake rate during the variable feeding period in group # 3 may reflect an increase in air temperature and a decrease in energy demands in the course of the season.

We propose that a perception of uncertainty triggers the hypothalamo-pituitary-adrenal (HPA)-axis, which results in increased circulating corticosterone. Evidence from other studies is consistent with this hypothesis. Increased corticosterone concentrations related to temporal unpredictability have been described in laboratory rats (Davis and Levine, '82). Uncertainty about feeding conditions possibly triggers corticosterone increase to induce 'escape-behavior' and the search for better conditions in songbirds (Silverin, '97; Wingfield and Ramenofsky, '97). Previous studies showed that adrenalectomy resulted in reduced exploration behavior in rats and that this behavior could be restored by endogenous implants of corticosterone (McIntyre, '76; Veldhuis et al., '82). Furthermore, injections of corticosterone increased locomotion in rats in a novel environment within minutes of injection. Such an effect was absent under familiar conditions (Sandi et al.,'96). In addition, implants of corticosterone to territorial white-crowned sparrows resulted in increased exploratory behavior and larger home ranges (Breuner,'98).

Also, small and acute elevations of circulating corticosterone have been shown to enhance spatial memory in rats in a spatial memory task (Luine et al.,'96). In addition, mountain chickadees (*Parus gambeli*) implanted with corticosterone were more competent in relocating cached seeds compared to controls (Saldanha et al., 2000). In the same species, Pravosudov and Clayton (2001) showed that limited and unpredictable feeding conditions improved the memory for caches with stored seed. This suggests that elevations of corticosterone to intermediate levels may promote the match of memory performance to unpredictably variable food supplies.

What is the function of elevated circulating corticosterone levels under temporarily variable food conditions in the life of red knots? Red knots spend the nonbreeding season on extensive mudflats in Western Europe where they feed in large flocks on buried bivalves. Red knots locate these prey items by probing in the mud with their tactile-sensitive bills (Piersma et al., '93, '98). Because shellfish are buried in the sediment, profitable feeding locations can only be located by intensive 'sampling.' Such prey items have a patchy distribution in the Wadden Sea (Piersma et al., '95). Rich patches of prey can change rapidly because of: (1) depletion by the shorebirds themselves (e.g., Goss-Custard, '77, '84);

(2) variable growth rates of individual shellfish (Piersma et al., '93); and (3) changes in burying depth (Reading and McGrorty, '78; Zwarts et al., '92; Piersma et al., '94). Such spatial and temporal variation in the density of prey would necessitate sustained exploration.

We predict that alterations in circulating levels of corticosterone may result from the variation in food availability in these natural habitats. In this way, costly forms of explorative behavior and/or enhanced memory performance are tailored to the needs. Incidentally, this also means that corticosterone concentrations may reflect how these birds evaluate their environment in terms of predictability. By measuring baseline corticosterone levels, we may be able to assay overall feeding conditions from the predators' perspective (cf. Wingfield et al., '97).

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